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Atherosclerosis and Lipoproteins

Long-Term Effects of Vitamin E, Vitamin C, and Combined Supplementation on Urinary 7-Hydro-8-Oxo-2'-Deoxyguanosine, Serum Cholesterol Oxidation Products, and Oxidation Resistance of Lipids in Nondepleted Men

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Abstract

Abstract—We studied the long-term effects of vitamins E and C and their combination on lipid peroxidation in vivo and in vitro. The Antioxidant Supplementation in Atherosclerosis Prevention (ASAP) trial is a double-masked placebo-controlled randomized clinical trial to study the effects of

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vitamin C (500 mg of slow release ascorbate per day), vitamin E (182 mg of RRR-α-tocopherol acetate per day), and the combination of both antioxidants. Lipid peroxidation measurements were carried out for 48 male participants at entry and at 12 and 36 months. Compared with placebo, vitamin E and the vitamin combination increased plasma lipid–standardized α-tocopherol during the first 12 months by 68.2% and 65.2% ($P<0.001$ for both), respectively, and reduced serum 7ß-hydroxycholesterol by 50.4% ($P=0.013$) and 44.0% ($P=0.041$), respectively. The net change of lipid standardized α-tocopherol was 63.8% after 36 months of vitamin E supplementation and 43.3% for the combination. Vitamin C supplementation elevated plasma total ascorbate level by 30.1% ($P=0.043$) in 12 months and by 91.1% ($P=0.001$) in 36 months. Neither vitamin E, vitamin C, nor the combination influenced the urinary excretion rate of 7-hydro-8-oxo-2'-deoxyguanosine or the antioxidative capacity of plasma. Vitamin E and the combination of vitamins E and C enhanced the oxidation resistance of isolated lipoproteins and total serum lipids. Our data indicate that long-term supplementation of nondepleted men with a reasonable dose of vitamin E alone or in combination with slow release vitamin C reduces lipid peroxidation in vitro and in vivo, whereas a relatively high dose of vitamin C alone does not.

Key Words: antioxidants • lipid peroxidation • oxidation resistance • 7-hydro-8-oxo-2'-deoxyguanosine • oxysterols

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Vitamins E[1 2 3 4] and C[5 6] are widely regarded as important dietary antioxidants. However, this concept is based on in vitro studies and on supplementation trials in which only measurements of lipid peroxidation ex vivo have been performed.[3] The evidence of the lipid peroxidation–inhibiting effects of vitamins E and C in vitro is plentiful.[1 2 3 4 5 6 7] In addition, oral vitamin E supplementation has consistently increased the oxidation resistance of isolated lipoproteins in vitro.[8 9 10 11 12 13] Vitamin C has had been shown to have a similar effect in a few[14 15 16] but not in all[17] studies. Vitamin C can also act as a pro-oxidant in certain conditions.[17 18] In theory, vitamin E could function as a mediator of lipid peroxidation if sufficient coantioxidants are not present.[19 20] However, this theory has not been tested in oral supplementation studies with in vivo measurements. There are no previous long-term placebo-controlled oral supplementation trials concerning the effects of these vitamins on lipid peroxidation in nonsmoking clinically healthy subjects.

The assessment of oxidative stress and lipid peroxidation in vivo in humans is problematic. 7-Hydro-8-oxo-2'-deoxyguanosine (8-oxodG), a repair product of oxidative damage to DNA, has been used as an indicator of intracellular oxidative stress.[21 22] Some of the preferred measures of lipid peroxidation in vivo are the cholesterol oxidation products.[23 24 25 26 27 28 29] In 1994,

we started a randomized-factorial, double-masked, placebo-controlled, long-term trial to study the effect of supplementation with vitamins E and C on atherosclerotic progression and the hypothesized pathways of the expected preventive effects. Measurements of in vitro and in vivo lipid peroxidation were carried out at entry and at 12 and 36 months in a subset of 48 male participants. The purpose of the present study was to present results concerning the effects of vitamin E and C supplementation on these measurements.

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Study Design and Supplements

The Antioxidant Supplementation in Atherosclerosis Prevention (ASAP) study was designed to test the main study hypothesis that the supplementation of 45- to 69-year-old smoking and nonsmoking men and postmenopausal women (520 subjects) with either 182 mg of RRR-α-tocopherol or 500 mg of slow-release ascorbic acid daily or both will retard the progression of common carotid atherosclerosis. ASAP is a clinical placebo-controlled double-masked trial with 2x2 factorial design with randomization in 4 balanced strata. Approximately half of the subjects were regular smokers (≥5 cigarettes per day) at screening. All subjects had hypercholesterolemia, defined as serum cholesterol of at least 5.0 mmol/L at screening. The study consisted of an 8-week placebo lead-in phase and a 3-year double-masked phase, for which the subjects were randomly allocated to either (1) 91 mg of RRR-α-tocopherol (Corresponding to 136 IU of vitamin E and 100 mg of RRR-α-tocopheryl acetate) twice daily, (2) 250 mg slow-release ascorbic acid twice daily, (3) both RRR-α-tocopherol and ascorbic acid in a single tablet (CellaVie, Ferrosan A/S), or (4) placebo only. The doses were chosen on the basis of pilot and kinetic studies.[17 22 30 31] The subjects were randomized separately in 4 strata of approximately equal size: (1) smoking men, (2) nonsmoking men, (3) smoking postmenopausal women, and (4) nonsmoking postmenopausal women. All subjects gave a written informed consent. The study protocol was approved by the Research Ethics Committee of the University of Kuopio.

The subjects came to baseline visits and were randomized between October 1994 and October 1995. The follow-up visits were exactly 12 and 36 months later to avoid the effects of seasonal changes. For the present study, extensive measurements were performed in a subset of 48 consecutive men at the baseline visit between July and October 1995 and at the 12-month and 36-month follow-up visits. Supplements were given, and returned tablets were counted at all these visits. The proportion of tablets used was 92.3%, 94.6%, 94.3%, and 92.3% at 12 months in the vitamin E group, vitamin C group, combination group, and placebo group, respectively. The respective proportions of tablets used at 36 months were 91.9%, 94.4%, 93,8%, and 94.6%.

Subjects were not entered into the trial if they had the following: premenopause or regular oral estrogen substitution therapy (women), regular intake of antioxidants, acetylsalicylic acid, or any other drug with antioxidative properties, severe obesity (body mass index >32 kg/m^2), type

1 diabetes, cataracts extracted bilaterally (making opacity assessment impossible), uncontrolled hypertension (sitting diastolic blood pressure >105 mm Hg), any condition-limiting mobility making study visits impossible, severe disease (shortening life expectancy), or other disease or condition worsening the adherence to the measurements or treatment.

Blood Sampling and Urine Collection

Subjects were instructed to abstain from eating for 12 hours and from ingesting alcohol for a week before blood sampling. After the subject had rested in a sitting position for 5 minutes, blood was drawn with Venoject vacuum tubes (Terumo). No tourniquet was used. Blood for lipoprotein fractionation and plasma total peroxyl radical–trapping antioxidant parameter (plasma TRAP) was collected in tubes containing EDTA and handled and measured immediately. Serum lipid oxidation measurement was performed immediately, and cholesterol, LDL cholesterol, HDL cholesterol, and triacylglycerol measurements were performed in batches every other day. Butylated hydroxytoluene serum samples for measurements of cholesterol oxidation products were immediately frozen to -70°C. Blood for α-tocopherol and ascorbic acid measurements was collected in tubes containing lithium and heparin, and plasma was stabilized with metaphosphoric acid for ascorbic acid determination and stored at -70° until used. A 24-hour urine sample was collected during the 24 hours preceding the visit to the laboratory for drawing blood.

Measurement of 8-OxodG

Urinary excretion of 8-oxodG was measured by 3D high-performance liquid chromatography (HPLC) and electrochemical detection in deep-frozen samples as previously described in detail for 24-hour urine samples.[22] The baseline and the 12-month samples for each person were measured in duplicate from the same batch.

Measurement of Cholesterol Oxidation Products

Measurements were performed in deep-frozen baseline and 12-month butylated hydroxytoluene serum samples. Concentrations of 9 cholesterol oxidation products were determined at the Karolinska Institute by isotope dilution mass spectrometry and the use of a deuterated internal standards.[23] These were 7α-hydroxycholesterol (7αOH), 7ß-hydroxycholesterol (7ßOH), 24-hydroxycholesterol, 25-hydroxycholesterol, 27-hydroxycholesterol, 7-oxocholesterol (7K), cholesterol α-epoxide (α-EPOX), cholesterol ß-epoxide (ß-EPOX), and cholestan-3ß,5α,6ß-triol (α-TRIOL).

Measurement of Oxidation Resistance of Isolated VLDL+LDL

VLDL and LDL were isolated in a combined fraction from fresh EDTA plasma by ultracentrifugation at baseline and at 12 and 36 months. Immediately after VLDL+LDL fraction separation, the EDTA and gradient salts were removed by gel permeation columns, and VLDL+LDL was exposed to copper-induced oxidation as previously described.[32] Time to maximal oxidation rate (lag time) and maximum reaction velocity (V_{max}) were determined.

Measurement of Serum Lipid Resistance to Oxidation

Lipoprotein resistance to oxidation in fresh serum at baseline and at 12 and 36 months was measured with a modification of the method described by Regnström et al.[33] Serum was diluted to a concentration of 0.67% in 0.02 mol/L PBS, pH 7.4. Oxidation was initiated by the addition of 100 μL of 1 mmol/L $CuCl_2$ into 2 mL of diluted prewarmed (30°C) serum. The formation of conjugated dienes was followed by monitoring the change in the 234-nm absorbance at 30°C on a Beckman Du 640I spectrophotometer equipped with a 6-position automatic sample changer.

Measurement of Plasma TRAP

Plasma TRAP was determined with a modification of the method of Metsä-Ketelä[34] as previously described[35] at baseline and at 12 and 36 months.

Measurement of Vitamins C and E and α-CEHC

Ascorbic acid and dehydroascorbic acid were determined in batches by HPLC.[36] The sum of ascorbic acid and dehydroascorbic acid (total ascorbic acid [TAA]) concentration in plasma was used for statistical analysis. Heparin plasma for α-tocopherol was extracted with ethanol and hexane and measured by reversed-phase HPLC.[32] The 24-hour urinary excretion of 1,5,7,8-tetramethyl-2(2'-carboxyethyl)-6-hydroxychroman (α-CEHC) was assessed by gas chromatography.[37]

Other Measurements

Serum total cholesterol and triacylglycerol concentrations were determined enzymatically (Konelab) with an autoanalyzer (Kone Specific, Konelab). Serum LDL cholesterol was precipitated by using polyvinyl sulfate (Boehringer-Mannheim) and calculated as the difference between total and supernatant cholesterol. Serum HDL cholesterol was measured after precipitation with magnesium chloride. All the measurements were performed at baseline and at 12 and 36 months. Dietary intake of foods and nutrients was assessed at baseline and at the 36-month study visit by using a 4-day food recording, which was completed in an interview by a dietitian.

Statistical Methods

Statistical analyses were carried out by using a statistical program software system (SPSS 9.0.1 for Windows). To separate the effect of α-tocopherol from that of serum lipids, lipid-standardized α-tocopherol was used in the statistical analysis.[38] The net change was calculated as the mean change in the supplemented group minus the mean change in the placebo group. Nonparametric Kruskal-Wallis 1-way ANOVA was used to compare the heterogeneity between the groups at baseline. Differences in changes between groups at 12 months and at 36 months were tested by the Mann-Whitney *U* test. The 95% CIs were calculated on the basis of *t* distribution. Differences in selected baseline characteristics of the study subjects between smokers and nonsmokers were tested by parametric Student *t* test or nonparametric Mann-Whitney *U* test. Normality of distributions was confirmed by the Kolmogorov-Smirnov test.

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Baseline Distributions

The main baseline characteristics of the study subjects are presented in Table 1. There were neither statistically significant nor biologically meaningful differences between the randomized groups. Serum total cholesterol and LDL cholesterol were higher than usual among Finnish men because of the criterion of hypercholesterolemia (serum cholesterol ≥5 mmol/L) at entry. The mean fiber intake (22.2 g/d) did not reach the Nordic Nutrition Recommendations[39] (25 to 35 g/d) but was precisely the same as reported earlier among adults in Finland.[40] The official saturated fatty acid recommendation (≈10 energy %) was not reached, with the average intake being 16.7 energy %.

View this table: **Table 1.** Baseline Characteristics of Study Subjects
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The study subjects consisted of 26 nonsmokers and 22 smokers distributed between groups, as shown in Table 1. Smokers had 30.5% (95% CI 10.9% to 42.0%) lower mean plasma TAA levels and significantly (P=0.039) shorter serum lipid oxidation lag times. Serum α-TRIOL was nearly significantly (P=0.051) lower in nonsmokers than in smokers. Other in vivo lipid peroxidation indicators tended to be nonsignificantly elevated in smokers. There was no difference between smokers and nonsmokers in either lipid-standardized plasma α-tocopherol, plasma α-tocopherol, oxidation resistance of VLDL+LDL, antioxidative capacity of plasma, or mean intakes of vitamin E, vitamin C, or fiber.

Effects of Supplementation on Plasma Total Vitamin C and E Concentrations

The mean plasma TAA and lipid standardized α-tocopherol concentrations declined significantly during the first 12 study months in groups that did not get proper supplementation. The mean plasma TAA was reduced by 28% in the placebo group and by 41% in the vitamin E group (Table 2). The respective decline in the lipid-standardized plasma α-tocopherol was 31% in the placebo group and 30% in the vitamin C group (Table 2). However, plasma α-tocopherol was increased slightly (by 1%) in the placebo group and reduced by only 0.6% in the vitamin C group. After 36 months of supplementation, the mean plasma TAA was reduced by only 5% in the placebo group and 2% in the vitamin E group (Table 2). Lipid-standardized plasma α-tocopherol declined by 26% in the placebo group and by 27% in the vitamin C group (Table 2), whereas plasma α-tocopherol was increased by 12% and by 6%, respectively.

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Table 2. Baseline Values and 12- and 36-Month Changes in Plasma Vitamin E and C and Lipid Peroxidation Measurements in 48 ASAP Participants Who Received Vitamin E, Vitamin C, Both Antioxidants, or Placebo

During the first 12 study months, the net changes of lipid standardized α-tocopherol in vitamin E and combination groups were 68.2% and 65.2%, respectively. During 36 months of supplementation, the net change was 63.8% in the vitamin E group and 43.3% in the combination group (Table 2). The mean increase of lipid-standardized α-tocopherol was greater in nonsmokers than in smokers after 12- and 36-month supplementation periods.

The mean plasma TAA concentration increased from 60.3 to 61.8 μmol/L (by 2%, P=0.043) in the vitamin C group and from 68.9 to 77.2 μmol/L (by 12%, P=0.071) in the vitamin E+C group during first 12 months. The net changes were 30.1% and 39.6%, respectively. The respective net changes after 36 months of supplementation were 91.1% and 49.9% (Table 2).

After 12 months of supplementation, the mean decrease of plasma TAA was nonsignificantly greater in nonsmokers than in smokers, whereas after 36 months, the mean increase of plasma vitamin C was greater in smokers (from 59.3 to 86.7 μmol/L) than in nonsmokers (from 80.6 to 97.3 μmol/L).

The 24-hour urinary excretion of the major metabolite of α-tocopherol, α-CEHC, was 1.9-fold (P=0.001) among men who received vitamin E (5.87±0.2.41 mg/d) compared with placebo (2.04±1.00 mg/d) and 2.4-fold (P=0.000) among men who received vitamin E+C (7.02±4.18 mg/d) compared with placebo. Among men who received vitamin E at 12 months, α-CEHC was significantly (P=0.03) greater in nonsmokers than in smokers. The correlation between lipid-standardized plasma α-tocopherol concentration and urinary α-CEHC excretion at 12 months was 0.62 (P<0.001).

Effects on DNA Oxidation Marker

Neither vitamin C, vitamin E, nor combined supplements had any statistically significant effect on the urinary excretion rate of 8-oxodG (Table 2).

Effects on In Vivo Lipid Peroxidation Markers

Supplementation with vitamin E reduced (net change) serum concentration of the principal cholesterol oxidation product, 7ßOH, by 50% (Table 2) and 7αOH by 35%. Compared with placebo, 12 months of vitamin C supplementation significantly reduced α-EPOX (P=0.049) and ß-EPOX (P=0.034). The significant net change of 7ßOH was 44% in the vitamin combination group.

Effects of Ex Vivo Lipid Peroxidation Markers

After 12 months of supplementation, the total serum lipid resistance to oxidation, measured as

lag time, was statistically significantly decreased in the combination group compared with the placebo group, whereas decreases in the other groups were not statistically significant. After 36 months of supplementation, vitamin E and the combination of vitamins E and C resulted in a significant increase in lag time. In the vitamin E group, lag time was increased by 8.5%, and in the vitamin E+C group, it was increased by 12.5%. Neither vitamin E nor vitamin C had any effect on $V_{max.}$ However, after a 12-month supplementation of vitamin E+C, the decrease of V_{max} was statistically significant compared with the placebo value.

Oxidation resistance of VLDL+LDL increased significantly after the 12- and 36-month supplementation periods in the vitamin E and combination groups compared with the placebo group. During first 12 months, the mean lag time increased by 27.7% in the vitamin E group and by 15.6% in the vitamin E+C group. The respective changes of lag time after 36 months were 27.2% and 21.4%. Vitamin C had no effect on lag time after either 12 or 36 months. V_{max} decreased significantly (by 26.5%) after 12 months of supplementation of vitamin E and by 22.0% after 36 months. Compared with placebo, vitamin C and combination supplementation had no statistically significant effect on V_{max}.

Neither vitamin E, vitamin C, nor combination supplementation had any significant effect on the antioxidative capacity of plasma (Table 2).

Discussion

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The present study is the first randomized placebo-controlled oral supplementation trial showing an in vivo lipid peroxidation–reducing effect of vitamin E and a combination of vitamins E and C in clinically healthy humans.

During the first 12 months, the mean plasma TAA and lipid standardized α-tocopherol concentrations declined significantly: plasma TAA in placebo and vitamin E groups and lipid-standardized α-tocopherol in placebo and vitamin C groups. Serum oxidation resistance, defined as lag time, and plasma TRAP changed accordingly in the first study year, even though the seasonal changes were avoided by arranging the follow-up visit exactly 12 months later than the baseline visit. These changes were probably due to changes in diet, which we did not assess at the 12-month study visit. After 36 months of supplementation, changes in placebo group were not statistically significant, and plasma concentrations of α-tocopherol and TAA increased significantly in the groups that got the respective vitamin supplements. The mean dietary intake of vitamins E and C, saturated fatty acids, and fiber did not change significantly in 36 months.

Even though there was no significant difference between smokers and nonsmokers in plasma α-tocopherol, at 12 months the α-CEHC excretion was significantly greater in nonsmokers than in

smokers in the vitamin E group. Generally, smokers suffer from continuous oxidative stress. They may excrete less α-CEHC because their α-tocopherol is used in antioxidative defense and is not degraded for excretion.

Vitamin E but not vitamin C increased the oxidation resistance of isolated atherogenic lipoproteins (VLDL+LDL). This difference is plausible on the basis of the lipoprotein separation process that eliminates vitamin C from the measurement. However, there are only a few oral supplementation studies concerning the effect of supplementation with ascorbic acid on lipid peroxidation, and the results are not congruent.[14] [15] [16] [17] Discrepancy in the results might be due to the difference in the lipoprotein separation procedure or further handling of the samples or may be simply due the difference in the methods used to measure lipid peroxidation.

The difference in change in lag time (VLDL+LDL oxidation) between vitamin E and the combination group was not significant after 12 and 36 months. Also, the 3-year supplementation of vitamin E did not result in longer lag times or lower V_{max} than 1-year supplementation; therefore, the maximum effect of vitamin E was achieved during the first year.

A surprising finding was that vitamin C did not improve the antioxidative capacity of plasma in either the 12-month or the 36-month follow-up. In a cross-sectional regression analysis of baseline data of the ASAP study, vitamin C and urate were the strongest determinants of plasma TRAP.[35] This is in accordance with a previous supplementation study of 5 men[41] and could be due to the nondeficient baseline plasma vitamin C levels of the present subjects.

The combined supplementation of vitamin E+C increased the oxidation resistance of total serum lipids more efficiently than supplementation of vitamin E or vitamin C alone in the 36-month follow-up. During the first 12 months, lag time decreased considerably in each group, except in the combined group. After 36 months of supplementation, the lag time increased in each supplemented group, as was expected. There seemed to be a synergistic interaction between vitamins C and E. Combined supplementation also decreased V_{max} significantly at the 12-month follow-up, whereas V_{max} increased among the other groups.

The lack of effect of both vitamins on the repair product of DNA oxidative damage is consistent with our previous finding from the shorter-term Multiple Anhoxidane Supplementation Intervention Study (MASI) study.[22] Supplementation with exogenous antioxidants does not appear to influence the intracellular oxidative stress, as indicated by the urinary excretion of 8-oxodG.

All previously published placebo-controlled human supplementation trials with vitamin E have been based mainly on in vitro measurements of oxidation resistance or susceptibility of isolated lipoproteins. Vitamin E supplements have, without exception, increased the oxidation resistance of LDL by 5% to 64% dose-dependently.[12] [13] In a study of Reilly et al,[42] vitamin E

supplementation at either 100 or 800 U/d failed to reduce the levels of an in vivo measurement of lipid peroxidation. In another uncontrolled study in 16 women and 6 men who were hypercholesterolemic, Davi et al[43] observed a 34% reduction in 12-hour urinary 8-epiprostaglandin $F_2\alpha$ excretion after 2 weeks of supplementation with 100 mg of d,l-α-tocopherol acetate and a 36% reduction after 2 weeks of supplementation with 600 mg.

According to a theory, α-tocopherol can promote lipid peroxidation in human LDL unless coantioxidants are present that eliminate the chain-carrying α-tocopheroxyl radical.[19] The only empirical support has come from in vitro experiments in which α-tocopherol depletion of LDL made it more resistant to oxidation during "low radical flux conditions" in the absence of other physiological antioxidants.[44] Our present findings are equivocal in this respect. Although vitamin E alone reduced the main cholesterol oxidation product equally to the vitamin combination, the oxidation resistance of total serum lipids was enhanced more by the vitamin combination than by either vitamin alone.

Cholesterol oxidation products have been demonstrated to be markers for cholesterol autoxidation in vivo.[23] [24] [25] [26] [27] [28] [29] Moreover, the major products of cholesterol autoxidation are the most commonly detected oxysterols in foods (7K, 7αOH, 7ßOH, α-EPOX, and ß-EPOX).[29] Various oxysterols have been detected in extensive amounts in human tissues and fluids, including human plasma, atherogenic lipoproteins, and atherosclerotic plaque.[29] Among the different oxysterols assayed, 7αOH, 24-hydroxycholesterol, and 27-hydroxycholesterol are formed enzymatically as products of cholesterol catabolism, whereas 7K and 7ßOH are not formed enzymatically in mammals. In the present randomized, double-masked, placebo-controlled, long-term trial, vitamin E and the combination supplement consistently reduced 7ßOH. On the basis of a cell culture study, Colles et al[45] concluded that 7ß-hydroperoxy-cholesterol, the labile precursor of 7ßOH, is the compound predominantly responsible for oxidized LDL–induced cytotoxicity. In our previous follow-up study, butylated hydroxytoluene serum 7ßOH concentration was a very strong predictor of atherosclerotic progression.[26] Also, Carpenter et al[46] found high concentrations of 7ßOH in early atherosclerotic lesions. Lizard et al[25] and Deckert et al[27] observed that 7ßOH inhibited arterial relaxation and had the greatest ability to induce apoptosis in endothelial cells of all cholesterol oxides measured. Recently, Zieden et al[28] reported that elevated plasma 7ßOH concentration is an indication of an increased in vivo lipid peroxidation. For these reasons, we regarded 7ßOH as the most important cholesterol oxidation measure. Vitamin C supplementation significantly reduced α-EPOX and ß-EPOX. However, the result might be artifactual, because this oxysterol is easily formed during sample processing and may be of dietary origin. There are no previous vitamin E supplementation studies in clinically healthy humans concerning its effects of oxysterols. Mol et al[47] supplemented diabetic persons and smokers with vitamin E (600 mg/d for 4 weeks) and found that α-TRIOL, 7K, and 7ßOH decreased in diabetic subjects and that α-EPOX decreased in smokers but not in the control subjects.

In conclusion, our present data show that in men who have usual plasma vitamin C and E levels, long-term oral supplementation with a reasonable dose of the natural isomer of α-tocopherol or with combined α-tocopherol and slow release vitamin C reduces lipid peroxidation in vivo and in vitro. Our findings further suggest that a relatively high dose of vitamin C alone does not have this effect.

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